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Survey of Endogenous Virus and *TVB** Receptor Status of Commercial Chicken Stocks Supplying Specific-Pathogen-Free Eggs

Henry Hunt,[A] Aly Fadly, Robert Silva, and Huanmin Zhang

United States Department of Agriculture, Agriculture Research Service, Avian Disease and Oncology Laboratory, 3606 East Mount Hope Road, East Lansing, MI 48823

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SUMMARY. Endogenous avian leukosis virus (ALVE) and the ALVE receptor (*TVB*S1*) status of six commercial chicken lines supplying specific-pathogen-free eggs were analyzed. All commercial chicken lines are certified free of the avian leukosis virus (ALV) by screening for expression of the p27 protein using the standard enzyme-linked immunosorbent assay. The commercial chicken lines A, E, and F contained replication competent ALVE inserts. Line A was fixed for ALVE21, and lines E and F were segregating for ALVE10. In addition, ALVE1 was detected in all the chicken lines. Chicken lines B, D, and F were essentially fixed for the *TVB*S1* allele that confers susceptibility to ALVE, whereas lines A, C, B, and E were resistant, containing either the *TVB*S3* or *TVB*R* alleles. The results show that lines selected to be ALV p27 negative give rise to two different genotypes. One genotype lacks the *TVB*S1* receptor for ALVE. Chicken lines with the *TVB*S1* negative genotype can retain replication competent endogenous virus inserts such as ALVE2, 10, or 21 and still display the p27 negative phenotype. These replication competent ALVE viruses are phenotypically p27 negative in the absence of the *TVB*S1* receptor because their chromosomal integration sites restrict transcription and subsequent production of the p27 protein and virus particles to levels below the detection limit. If the *TVB*S1* receptor is present, the limited production of ALVE virus particles reinfects and integrates into more productive chromosomal locations in the cell. Increased production of infective virus particles and detectable levels of p27 follow this reinfection and integration into more active regions of the cells genome. The other genotype observed in the commercial lines retains the ALVE receptor (*TVB*S1*) but either lacks replication competent inserts or expresses the envelope encoded protein from defective inserts such as ALVE3 or ALVE6. In this phenotype, the env-coded glycoprotein encoded by the defective inserts binds to the *TVB*S1* receptor and blocks the reinfection of the replication competent ALVE virus. This receptor interference stops reinfection and subsequent production of detectable virus particles and the p27 protein. Mixtures of different p27 negative phenotypes can result in the p27 positive phenotype and ALVE virus production. For example, mixtures of ALVE receptor positive (*TVB*S1*) but ALVE negative (p27 negative and envelope negative) chick embryo fibroblasts (CEFs) with fibroblasts that are receptor negative but ALVE positive could generate cells expressing high levels of p27 and ALVE virus. In this situation, the undetectable levels of ALVE virus from the receptor negative CEFs would infect and integrate into the receptor positive CEFs and produce detectable levels of ALVE virus. The implications of these findings for vaccine manufacturers and regulatory agencies are discussed.

RESUMEN. Estudio sobre la presencia de virus endógenos y receptores para el virus de leucosis aviar (*TVB**) en parvadas comerciales productoras de huevos libres de patógenos específicos.

Se analizó la presencia de virus endógenos de leucosis aviar y de receptores para virus endógenos de leucosis aviar (*TVB*S1*) en seis líneas comerciales productoras de huevos libres de patógenos específicos. Todas las líneas comerciales son certificadas como libres del virus de leucosis aviar utilizando la prueba estándar de inmunoensayo asociado a enzimas para la detección de la expresión de la proteína p27. Las líneas comerciales A, E y F contenían insertos de virus endógenos de leucosis aviar con capacidad de replicación. La línea A contenía el virus endógeno de leucosis aviar 21 de manera constitutiva y las líneas E y F segregaban el virus endógeno de leucosis aviar 10. Adicionalmente, el virus endógeno de leucosis aviar 1 se detectó en todas las líneas. Las línea B, D y F contienen el alelo *TVB**S1 que confiere susceptibilidad a virus endógenos de leucosis aviar, mientras que las líneas A, C, E son resistentes, pues contienen el alelo *TVB**S3 o el *TVB**R. Los resultados muestran que las líneas seleccionadas como negativas a p27 dan origen a dos genotipos diferentes. Un genotipo que no presenta el receptor para virus endógenos de leucosis aviar (*TVB**S1). Las líneas con el genotipo negativo al *TVB**S1 pueden retener insertos de virus endógenos capaces de replicación, tales como los virus endógenos de leucosis aviar 2, 10 o 21 y aun así presentar un fenotipo p27 negativo. En la ausencia del receptor *TVB**S1, estos virus endógenos de leucosis aviar capaces de replicación son fenotípicamente negativos a p27, debido a que sus sitios de integración cromosomal restringen la trascripción y la subsecuente producción de partículas virales y de la proteína p27 a niveles por debajo de los limites de detección. Si el receptor *TVB**S1 está presente, la producción limitada de partículas de virus endógenos de leucosis aviar reinfecta la célula y se integra en segmentos cromosomales mas productivos. Incrementos en la producción de partículas virales infecciosas y de niveles detectables de p27, siguen a la mencionada reinfección e integración en regiones mas activas del genoma de la célula. El otro genotipo observado en las líneas comerciales, retiene el receptor *TVB**S1 pero carece de insertos capaces de replicación o expresan la proteína de envoltura codificada por insertos de replicación defectiva, tales como virus endógenos de leucosis aviar 3 y 6. En este fenotipo la glicoproteína de envoltura codificada por los insertos defectivos, se une al receptor *TVB**S1 y bloquea la reinfección de virus endógenos de leucosis aviar capaces de replicación. Esta interferencia de los receptores paraliza la reinfección y la subsecuente producción de niveles detectables de partículas virales y de proteína p27. Mezclas de diferentes fenotipos negativos a p27 pueden resultar en el fenotipo positivo a p27 y en la producción de virus endógenos de leucosis aviar. Por ejemplo, la mezcla de fibroblastos de embrión de pollo que contienen virus endógenos de leucosis aviar positivos al receptor *TVB**S1 pero que son negativos a virus endógenos de leucosis aviar (negativos a las proteínas p27 y de envoltura), con fibroblastos que son negativos al receptor pero positivos a la presencia de virus endógenos, podría generar células expresando altos niveles de p27

[A]Corresponding author. E-mail: henry.hunt@ars.usda.gov

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y de virus endógenos de leucosis aviar. En esta situación, los niveles no detectables de virus endógenos de leucosis aviar proveniente de los fibroblastos de embrión de pollo negativos al receptor podrían infectar e integrarse en los fibroblastos de embrión de pollo positivos al receptor y producir niveles detectables de virus endógenos de leucosis aviar. Se discuten las implicaciones de estos hallazgos para los fabricantes de vacunas y las agencias regulatorias.

Key words: retrovirus, poultry, endogenous, receptor, pathogen free, chicken

Abbreviations: ADOL = Avian Disease and Oncology Laboratory; ALV = avian leukosis virus; ALVE = endogenous avian leukosis virus; CEF = chick embryo fibroblast; EAV = endogenous avian retrovirus; ELISA = enzyme-linked immunosorbent assay; Env = envelope; LTR = long terminal repeat; PCR = polymerase chain reaction; Pol = polymerase; RFLP = restriction fragment length polymorphism; SBI = specific binding index; SNP = single-nucleotide polymorphism; SPF = specific-pathogen-free

The genomes of most animals contain a series of retrotransposons derived from the germline integration of retroviruses. In chickens, there are at least four families of retrotransposons. The endogenous avian retrovirus (EAV) family was apparently derived from ancient virus integration events (10). The *EAV*s represent highly deleted viral genomes lacking regions encoding the reverse transcriptase enzyme (polymerase or Pol) and envelope (Env) genes necessary for provirus replication and transmission (11,14). The DNA sequence of the *EAV* long terminal repeats (LTRs) are highly diverse, presumably due to random degeneration of these sequences over evolutionary time, suggesting their ancient integration status.

Two other endogenous retrovirus families called *ART-CH* (17,23) and *ev/J* (8,26) or *EAV-HP* (33) are derived from more recent integrations, and they are closely related to the exogenous avian leukosis virus subgroup J (ALV-J). These retrovirus integrations also lack regions encoding Pol; thus, they are defective in their ability to replicate (26). The *ART-CH* and *EAV-HP* loci do produce defective RNA transcripts that apparently do not translate viral protein products. These defective transcripts do contain packaging signals, and the possibility exists that they could be packaged into virus particles with the aid of a helper virus. More importantly, these defective templates recombined with exogenous ALV to create the new ALV-J virus.

The endogenous avian leukosis virus (*ALVE*) family is the most characterized of the endogenous viruses in chickens (for review, see 13). The *ALVE* loci are closely related to exogenous viruses displaying approximately 80% homology at the DNA level with ALV subgroups A through D (9). The *ALVE* series includes at least 23 loci consisting of both defective and nondefective retroviral inserts (1,19,20,25,29,31,32,34,35,36,40). The nondefective loci are capable of producing infectious virus particles, and they are the only endogenous virus family that can transmit horizontally without the aid of helper viruses. Research has shown that expression of ALVE proteins during ontogeny renders the chicken's immune system tolerant to exogenous ALV viruses. Chickens rendered tolerant by *ALVE* produce neutralizing antibody weeks later and with severalfold less titer than their nontolerant counterparts.

The ability of some *ALVE* loci to transmit horizontally makes it imperative that professionals in the poultry industry understand the biology and consequences of this important genetic complex. The biology of *ALVE* transmission is intimately associated with the cellular receptor complex *TVB**. The *TVB** genetic complex is a series of three alleles (*TVB*S1*, *TVB*S3*, and *TVB*R*) that encode the cellular receptors for three subgroups of the ALVs: the exogenous ALV subgroups B and D along with the endogenous ALVE virus. The *TVB*S1* allele encodes the receptor for ALV subgroups B, D, and E. The receptor encoded by the *TVB*S3* allele binds ALV-B and D but not E, and *TVB*R* encodes a truncated receptor incapable of binding all three ALV subgroups. The segregation of *ALVE* and *TVB** loci in commercial chicken lines used to supply specific-pathogen-free (SPF) eggs to the poultry industry creates a complex interrelationship of phenotypes. The segregation of these phenotypes produces mixtures of SPF chick embryo fibroblasts (CEFs), some capable of propagating endogenous ALVE and some resistant to ALVE infection. The ability to propagate ALVE virus in SPF CEFs is an undesirable trait for live poultry vaccine manufactures and the end users of these vaccines. Conversely, CEFs with the ability to propagate ALVE are valuable tools used by vaccine manufactures and regulatory agencies for monitoring undesirable ALVE contaminants in poultry biologicals. Unfortunately, commercial SPF providers do not offer choices with different genetic and phenotypic characteristics specifically designed for these purposes. This is primarily due to the complicated nature of the *ALVE*/*TVB** interaction and the lack of simple techniques for selection of genetic stocks with the desirable phenotypes.

New reagents and procedures have simplified the analysis of the *ALVE* and *TVB** loci. These new techniques, coupled with standard techniques, were used to investigate the *ALVE* and *TVB** profiles of six commercial lines of chickens commonly used to supply SPF eggs to poultry vaccine manufactures, poultry breeders, and agencies that regulate poultry biologicals. This article reviews ALVE biology, reports the *TVB** and ALVE loci status of six commercial SPF lines, and discusses their implications for the poultry industry. Finally, we propose that SPF suppliers produce two different genetically defined SPF products, one product for live poultry vaccine manufacturers and one product for biologicals testing. The SPF lines for the production of live poultry vaccines would lack both the replication competent *ALVE* loci and the *TVB** allele capable of binding ALVE (*TVB*S1*). These CEFs would be resistant to ALVE infection and would ensure that live poultry vaccines be free of replication competent ALVE. The other SPF product would be selected for biologicals monitoring, and it would lack all *ALVE* loci producing ALVE proteins but retain the *TVB** receptor for *ALVE* (*TVB*S1*). Importantly, these CEFs would not produce any ALVE proteins interfering with the susceptibility, replication, and detection of ALVE virus.

MATERIALS AND METHODS

Nomenclature. The genome mapping effort with the resulting chicken genome database (CHICKBASE) has generated the need for uniform nomenclature compatible with computer search routines. The necessity for a uniform, computer friendly coding system has changed the symbols used in prior literature to denote the endogenous virus loci and their cellular receptors. In the past, loci for the *ALVE* virus family were designated by *ev* followed by a number representing the order in which they were discovered such as *ev*-1 or *ev*-3. The new nomenclature uses the descriptive letters *ALVE* followed by the locus number, such as *ALVE*1 or *ALVE*3. As in the old nomenclature system, when referring to genetic loci, the symbol is italicized and when referring to the protein product expressed from the locus the symbol is not italicized.

A single locus such as *TVB** that contains multiple alleles was previously referred to using superscripts, for example, the *TVB**S1 allele

Table 1. Summary of the molecular characteristics of published endogenous virus inserts and the ADOL chicken lines used as controls.

ALVE locus[A]	PCR test[B]	Restriction fragment size[C]		*ALVE* phenotype[D]	ALVE receptor phenotype and ALVE status of representative ADOL lines[E]						
		*Bam*HI	*Sac*I		TVB*S1	TVB*S1/TVB*R	TVB*R	TVB*S1	TVB*S1	TVB*S3	TVB*S3/TVB*S1
1	*	5.2	9.5	Gag−/+, Env−/+	6_3	100B	7_2	15B1	$15I_5$	RH-C	
2	*	**8.2**	**6**	**EV+**		**100B**	**7_2**				
3/B7	*	7.3	6.3	Gag+, Env+	**6_3**						
4	*	7.3	8.7	Gag−, Env−							
5		13	19	Gag−, Env−							
6	*	4.4	21	Gag−, Env+					$15I_5$		
7	*Z ch	**7.6**	**13**	**EV+**						**RH-C**	
8/B2	*	23	18	Gag−, Env−							
9	*	11	23	Gag−, Env+							
10		**14**	**21**	**EV+**					**$15I_5$**	**RH-C**	
11		**-**	**13**	**EV+**							
12/B5	*	**-**	**8.1**	**EV+**							
13		-	-	-							
14		**15**	**9.5**	**EV+**							
15	*	21.7	4.3	Gag−, Env−				15B1	$15I_5$		
16	*	-	5.6	Gag−, Env−							
17		-	11	-							
18		25	10.5	EV+							
19		9.8 or 18.0	7.6	-							
20		9.8 or 18.0	8.1	-							
21	*	**20**	**9.2**	**EV+**							**0.44–21**
22		-	5.5	-							
23		6.1	4.2	Unknown							
B1	*	3.2	20	Unknown							
B3		7	13.5	Unknown							
B4		8	9	Unknown							
B6	*	11.2	7.8	Unknown							
B8		4	4.3	Unknown							
B9		7.7	29.9	Unknown							
B10		5	20.4	Unknown							
B11		6.8	11.5	Unknown							
B12		21.2	7.5	Unknown							

[A]The ALVE loci of White Leghorns are numbered 1–23; ALVE loci described in broiler lines are listed as B1–B12 (19,20,23,27,32,34,38).
[B]An asterisk (*) indicates there is a diagnostic PCR test for the locus (4,5,6,7,16).
[C]Southern blot fragment size in kilobases.
[D]The ALVE phenotype Gag indicates the presence (+) or absence (−) of a positive p27 ELISA result, Env indicates the expression of the envelope protein, and EV+ indicates the locus has the potential to produce infectious virus particles. The ALVE1 locus is a full-length endogenous virus but it is normally silenced; under certain conditions such as treatment with methylation inhibitors, ALVE1 can produce defective virus particles (12).
[E]For a review of the ADOL chicken lines used as controls, see Bacon *et al.* (3).

was designated *tvb*^{S1}. This nomenclature was changed by replacing the superscripts with an asterisk to denote alleles of a locus. Thus, *tvb*^{S1} is now referred to as *TVB*S1*, and the protein product produced from this locus is designated TVB*S1. We adhere to this new nomenclature throughout this discussion.

Chicken lines. *Reference lines*. Reference lines for the analysis of *ALVE* and *TVB** were maintained at Avian Disease and Oncology Laboratory (ADOL) and consisted of lines 6_3, 7_2, 100B, 15B1, $15I_5$, line 0, 0.44-VB*S1-EV21, and RH-C. For a review on the origin, maintenance, and genetic characteristics of these lines, see Bacon et al. (3). Table 1 lists the *ALVE* and *TVB** characteristics relevant to each line used in this analysis.

Commercial SPF lines. Six White Leghorn chicken lines, coded A through F, were kindly provided by the commercial breeders. Both male ($n = 8$) and female ($n = 10$) individuals were analyzed for lines A, B, C, and D. The sex of lines E ($n = 10$) and F ($n = 10$) were unknown. Blood samples were drawn in heparinized syringes, immediately cooled, and shipped on ice overnight to ADOL.

***ALVE* loci analysis.** *Restriction fragment length polymorphism (RFLP) analysis*. The methods for the preparation of DNA from whole blood samples and the Southern blotting procedure were as described previously (24). Briefly, DNA was extracted using the phenol chloroform method, digested with either *Sac*I or *Bam*HI restriction enzymes (New England Biolabs, Beverly MA), electrophoresed overnight in 0.6% agarose, and transferred by capillary action to nylon membranes (28). The *ALVE* LTR fragment (564 bp) (27) was labeled using a ^{32}P-nucleotide mix (New England Nuclear, Boston, MA) and the Prime-It II random priming kit (Strategene, La Jolla, CA) using the manufacturers' instructions. The labeled LTR probe was hybridized to the nylon membranes at 55 C overnight and washed three times at 65 C. The labeled membrane was then developed using Kodak XR film (Eastman Kodak, Rochester, NY). The different *ALVE* loci were identified by the size characteristics of the *Sac*I and *Bam*HI banding patters given in Table 1.

Polymerase chain reaction (PCR) analysis. The PCR diagnostic test for *ALVE*1, 2, 3, 4, 6, 7, 15, and 21 was used to confirm the results of the RFLP analysis described above. The locus specific *ALVE* nucleotide primers and PCR conditions were essentially as described previously (4), with the following modifications. The original multiplex PCR procedure uses three PCR primers to determine occupied and

unoccupied *ALVE* chromosomal integrations in one PCR reaction. This multiplex primer array was replaced by performing two separate PCR reactions, one reaction to amplify the occupied site and the other reaction to amplify the unoccupied site.

TVB* locus analysis. *R2 antibody analysis*. The R2 flow cytometric assay described previously (2) was performed to determine the *TVB** receptor status and the presence of subgroup E virus particles in the chicken sera. The R2 antibody detects the ALV-E virus when bound to its cell surface receptor (*TVB*S1*). Thus, chickens that test positive for R2 antibody have the *TVB*S1* receptor and produce subgroup E virus particles; this is referred to as the direct R2 assay. Chickens that are negative for the direct R2 assay may lack either the receptor, the env-coded glycoprotein, or both. To distinguish between these possibilities, cells from animals testing negative for R2 are incubated with serum known to contain subgroup E virus particles and then they are retested for R2 reactivity. This test, referred to as the indirect R2 assay, is positive only if the chicken has the *TVB*S1* receptor. If the cells are negative for R2 after being incubated with virus (indirect R2 assay), then the serum from the R2 negative chicken is mixed with control cells known to be *TVB*S1*. If the serum renders the cells positive for R2, the chicken is shedding subgroup E virus particles into its serum; this is referred to as the serum R2 assay. Chickens that are negative for all three tests are receptor negative, and they do not produce subgroup E virus particles. Test samples with a specific binding index (SBI) greater than 1.33 were considered positive (2).

PCR-RFLP assay. The PCR coupled with RFLPs assay used to detect the various isoforms of the *TVB** alleles was performed as described previously (37). Briefly, PCR products designated TVB303 (forward 5′-ACC CCT TCT TGC AGG CAC CTA TGA, reverse 5′-GGA TGC TGT GCT GCG TGG AGA) and TVB202 (forward 5′-GGT AAG GCA GTC ACA AGC ATC ACT C, reverse 5′-TAC TCG TCT TTC TTA CAT GGG AGG CTC T) were amplified and digested using endonuclease enzymes *Nla*III and *Xba*I, respectively. The resulting electrophoretic patterns identify the *TVB** allele status.

RESULTS

***ALVE* locus analysis.** In White Leghorn chickens, there are at least 23 *ALVE* loci that have been characterized (1,19,20,25,29,31, 32,34,35,36,40). Table 1 summarizes those loci commonly found in commercial chicken lines and lists the *TVB** status of various chicken lines maintained at ADOL and used as controls to analyze the *ALVE* and *TVB** status of the lines producing commercial SPF eggs. The *ALVE* loci range in size from a single LTR region such as *ALVE*15 to full-length retrovirus genomes, including *ALVE*2, 7, 10, 11, 12, 14, and 21. Southern blot analysis was the primary tool used to identify the *ALVE* inserts in the SPF breeder lines. When available, PCR analysis was used to confirm the Southern blot analysis. A sample of the Southern blot analysis is presented in Fig. 1. When available, PCR analysis was used. The Southern blot analysis clearly shows a complex RFLP pattern in some lines with some banding patterns not represented in the published literature. The unidentified bands may represent unnamed *ALVE* loci or could arise from known loci with genomic polymorphisms (single-nucleotide polymorphisms; SNPs) producing different restriction endonuclease patterns in the DNA surrounding the insert. For whatever reason, these unidentified bands were labeled A, B, C, and D for this discussion. The PCR tests for *ALVE* correlated with the Southern blot data, and they identify either the homozygous or heterozygous nature of *ALVE* inserts. There were two chickens in which the Southern blot RFLP and the PCR data did not correlate: Female line A #3 (A ♀ 3) was clearly positive for the *ALVE*6 RFLP pattern by Southern blot analysis but negative for the *ALVE*6 PCR test, and female line B #3 (B ♀ 3) displayed the *ALVE*1 RFLP pattern but it was negative for *ALVE*1 by PCR. These discrepancies are not due to trivial technical errors, because the same results were obtained repeatedly. Furthermore, PCR and RFLP patterns for *ALVE*1, 3, and 21 correlated using the same A ♀ 3 DNA and the B ♀ 3 DNA was positive for the *ALVE*1 negative primer set, indicating that DNA integrity was not a factor for the discrepancies between the RFLP and PCR analysis. The results indicate that lines A, E, and F contain full-length inserts (*ALVE*10 and *ALVE*21) capable of producing ALVE infectious virus particles. The *ALVE*1 endogenous virus is present in all the lines and under most circumstances is silent, however, it can be induced to produce an infectious virus particle.

***TVB** locus analysis.** The *TVB** receptor status of the lines were analyzed using R2 antiserum and PCR-RFLP techniques. The direct R2 assay is positive if the chicken has the *TVB*S1* receptor (S1+) and expresses the ALVE env-coded glycoprotein (env+). A negative direct R2 result cannot distinguish between the absence of the *TVB*S1* (S1−) allele or the lack of env-coded glycoprotein expression (env−). To distinguish between the S1− or env− phenotype, the cells are analyzed using an indirect R2 assay in which the cells are mixed with control serum known to contain the env-coded glycoprotein protein. A negative indirect R2 assay is the result if the sample lacks the S1 receptor. A third R2 assay, the serum R2 assay, is used to determine whether the chickens serum contains the ALVE env-coded glycoprotein. In the serum R2 assay, the chickens' serum is mixed with control cells known to express the *TVB*S1* allele but lack env-coded glycoprotein expression (S1+/env−). If the test serum turns the S1+/env− cells positive using the R2 assay, the chicken expresses the ALVE env-coded glycoprotein (env+). Using the direct, indirect, and serum R2 assays, the *TVB*S1* status and the expression the ALVE env-coded glycoprotein can be determined. The results of the R2 assays are presented in Table 2 along with the resulting conclusions expressed as either positive or negative for the *TVB*S1* allele and the ALVE env-coded glycoprotein. In one sample, B ♀ 1, the direct R2 assay was positive indicating the presence of both the S1 allele and the env protein; however, the serum R2 assay was negative, indicating this chicken did not express the env protein. Because the results from this sample were indetermined, it was given the ID label in Table 2.

The PCR-RFLP assay detects the SNP that varies between the S1, S3, and R allelic forms of the *TVB** receptor. This assay determines the heterozygous or homozygous nature of the *TVB** alleles, and it is shown in the *TVB** SNP column in Table 2. The *TVB** status determined by the R2 and PCR-RFLP techniques correlated for lines B, C, D (females), E, and F; however, the two assays did not correlate in lines A and D (males). All the samples in line D, both males and females, tested *TVB*R/**R (S1−) by the *TVB** SNP assay. The R2 assay identified samples from the females of line D as S1 negative, but the males were positive. The serum R2 assay indicates the absence of an env-coded glycoprotein in line D, and this correlates with the Southern blot analysis identifying *ALVE* inserts that produce the env− phenotype (see Table 1). Thus, there seems to be a male specific antigen in line D that cross-reacts with the R2 antiserum accounting for the *TVB*S1* false-positive results. Line A was positive by the direct and serum R2 assays, indicating a S1+/env+ phenotype. The env+ phenotype correlates with the Southern blot and *ALVE* PCR analysis that indicate this line is fixed for *ALVE*21 and *ALVE*3, both of which produce the env-coded glycoprotein. However, line A tested S1− by the PCR-RFLP SNP assay, bringing the direct R2 results into question. The PCR-RFLP assay has proven highly reliable in determining the *TVB** allele phenotype; thus, we conclude the direct R2 is a false-positive result.

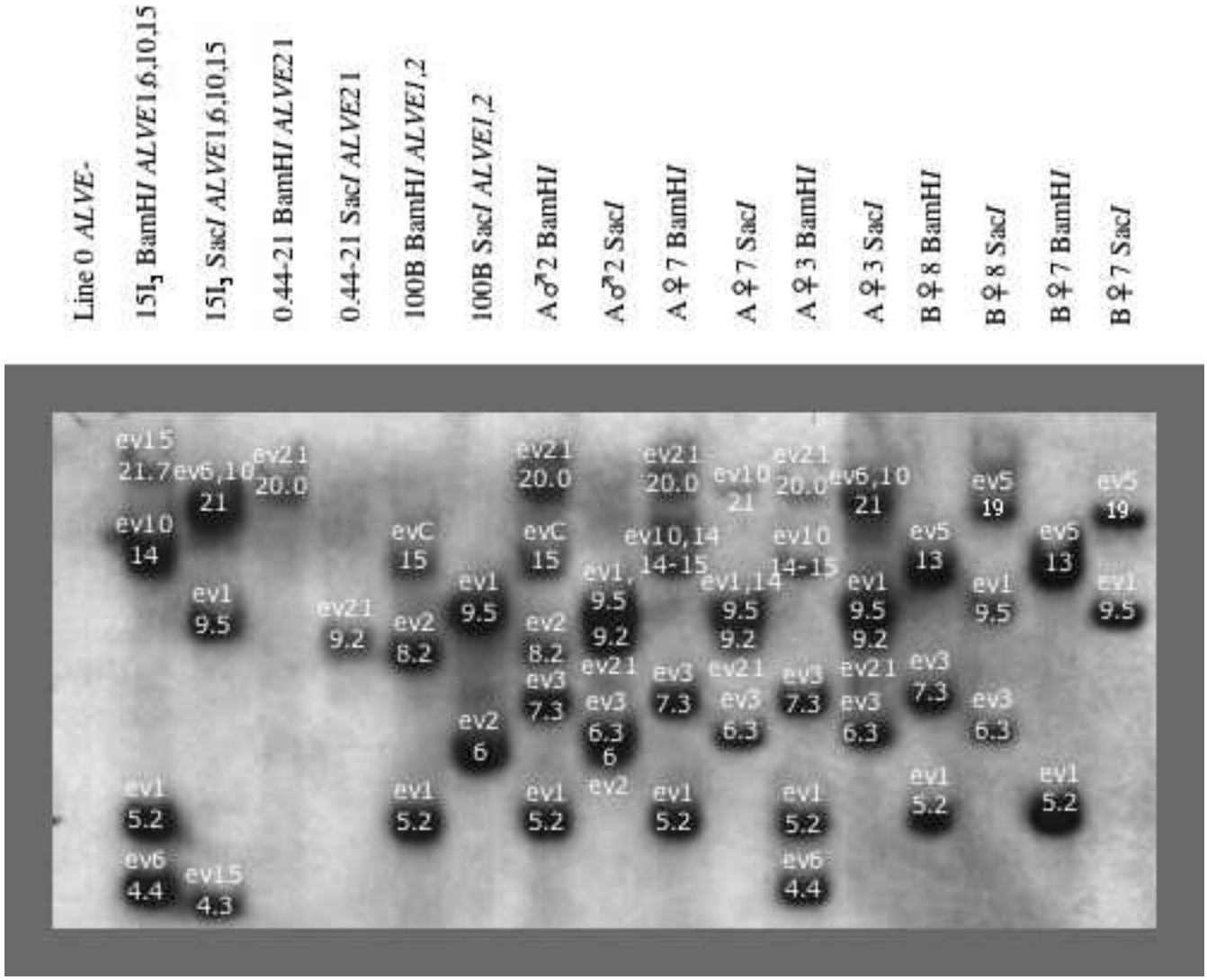

Fig. 1. Typical Southern blot used to identify the *ALVE* loci in the six commercial lines. ADOL lines 0, $15I_5$, 0.44–21, and 100B were routinely used as controls for each Southern blot to as an aid in determining the *ALVE* RFLP size and patterns of the commercial lines. Where banding patterns did not match any published RFLP pattern, they were labeled evA to D (see text).

DISCUSSION

Six commercial chicken lines that produce SPF fertile eggs were screened for their endogenous virus profile and the *TVB** alleles encoding the receptor for endogenous virus. These lines supply the bulk of the CEFs used to produce poultry biologicals, such as the Marek's disease vaccine as well as cells used by regulatory agencies for screening vaccine lots for various types of biological contamination. Lines A, E, and F contained replication competent *ALVE* inserts. Line A was fixed for *ALVE*21, and lines E and F were segregating for *ALVE*10. In addition, *ALVE*1 was detected in all lines. Lines B, D, and F were essentially fixed for the *TVB*S1* allele that confers susceptibility to *ALVE*, whereas lines A, C, B, and E were resistant, because they contained either the *TVB*S3* or *TVB*R* alleles. The importance of the *ALVE* virus and *TVB*S1* receptor segregation is illustrated by the chicken line 0.44-VB*S1-EV21. This line is fixed for the *ALVE*21 (ev21) locus but segregating for the *TVB*S1* and *TVB*S3* receptor alleles. Line 0.44-VB*S1-EV21 chickens that are *TVB*S3* homozygous are negative by the p27 enzyme-linked immunosorbent assay (ELISA) assay, indicating that the *ALVE*21 in this genetic background is essentially silent. However, in the context of the *TVB*S1* receptor, the 0.44-VB*S1-EV21 chickens are p27 positive, indicating the *ALVE*21 virus is active and producing virus proteins and infectious virus particles. The virus-positive nature of the *ALVE*21/*TVB*S1* combination is the result of a nearly silenced *ALVE*21 locus. The *ALVE*21 locus produces a small amount of *ALVE*21 virus that is undetectable by p27 ELISA assay. In the context of the *TVB*S1* receptor, this small amount of virus can re-enter the cells and integrate into a more active region of the genome, increasing the level of *ALVE*21 virus production to the level that is detectable by the p27 ELISA assay (for a review, see 13). This situation becomes complicated when other endogenous loci are present, for example, if *ALVE*3 is included with *ALVE*21 and the *TVB*S1* allele, the chicken will be negative for the p27 ELISA assay due to blockage or receptor interference induced by the env-coded glycoprotein produced by the *ALVE*3 locus.

All SPF chicken lines are routinely screened to be negative for the p27 ELISA assay. Indeed, our screening indicates that the line was fixed for *ALVE*21 was also fixed for the *TVB*S3* and *TVB*R* alleles. In addition, this line was fixed for the *ALVE*3 locus, which would induce receptor interference for the *ALVE*21 virus by producing the env-coded glycoprotein. The *TVB** receptor status along with the

Table 2. Results of the R2, TVB SNP, Southern blot, and ALVE PCR analysis of commercial SPF chicken lines.

	R2 *TVB* analysis[A]					Molecular analysis of *ALVE* loci								
							ALVE PCR primer sets[D]							
Sample no.	Direct R2 assay	Indirect R2 assay	Serum R2 assay	Conclusion from the direct, indirect and serum R2 assays[B]	*TVB* SNP	Southern blot[C]	*ev1+*	*ev1−*	*ev21+*	*ev21−*	*ev2+*	*ev6+*	*ev3+*	*ev3−*
Control line														
Ev6	DNA only	ND	ND		ND	ND	ND	ND	ND	ND	ND	+	ND	ND
Line 0	−	−	−	S1−/env−	*S3S3*	−	−	+	−	+	−	−	−	+
15B1	−	+	ND	S1+/env−	*S1S1*	ND	ND	ND	ND	ND	ND	ND	ND	ND
EV21+	+	ND	+	S1+/env+	*S1S3*	21	−	+	+	+	−	ND	ND	ND
6_3					*ND*	1,3								
7_2	−	−	−	S1−/env−	*RR*	ND	−	+	−	+	+	ND	ND	ND
15-I_5	+	ND	ND	S1+/env+	*S1S1*	1,6,10,15	+	−	−	ND	−	+	−	+
RFS 3313	−	+	−	S1+/env−	*S1S3*	ND	ND	ND	ND	ND	ND	ND	ND	ND
RFS 3372	−	+	−	S1+/env−	*S1S3*	ND	ND	ND	ND	ND	ND	ND	ND	ND
100B	+	ND	ND	S1+/env+	*S1R*	1,2	+	−	−	+	+	−	ND	ND
SPF line														
A ♀ 1	(+)	ND	+	S1+/env+	ND	ND	+	−	+	+	+	+	+	−
A ♀ 2	(+)	ND	+	S1+/env+	ND	ND	+	−	+	+	−	+	+	−
A ♀ 3	(+)	ND	+	S1+/env+	*S3R*	1,3,6,10,21,C	+	−	+	+	−	− ?	+	−
A ♀ 4	(+)	ND	+	S1+/env+	ND	ND	+	−	+	+	−	+	+	−
A ♀ 5	(+)	ND	+	S1+/env+	*S3R*	1,3,6,21,C	+	−	+	+	−	+	+	−
A ♀ 6	(+)	ND	+	S1+/env+	S3R	ND	+	−	+	+	−	+	+	−
A ♀ 7	(+)	ND	+	S1+/env+	*S3R*	1,3,5,21	+	−	+	+	−	−	+	−
A ♀ 8	(+)	ND	+	S1+/env+	ND	ND	+	−	+	+	+	+	+	−
A ♂ 1	(+)	ND	+	S1+/env+	ND	ND	+	−	+	+	−	+	+	−
A ♂ 2	(+)	ND	+	S1+/env+	*S3S3*	1,2,3,21,C	+	−	+	+	+	−	+	−
A ♂ 3	(+)	ND	+	S1+/env+	S3S3	ND	+	−	+	+	−	+	+	−
A ♂ 4	(+)	ND	+	S1+/env+	ND	ND	+	−	+	+	−	+	+	−
A ♂ 5	(+)	ND	+	S1+/env+	ND	ND	+	−	+	+	−	+	+	−
A ♂ 6	(+)	ND	+	S1+/env+	*S3R*	1,3,21,C	+	−	+	+	−	−	+	−
A ♂ 7	(+)	ND	+	S1+/env+	ND	ND	+	−	+	+	+	+	+	−
B ♀ 1	(+)	ND	−	?	*S1S1*	1,5	+	+	ND	ND	−	ND	−	+
B ♀ 2	+	ND	+	S1+/env+	ND	ND	+	+	ND	ND	−	ND	+	+
B ♀ 3	−	+	−	S1+/env−	*S1S1*	1,5	− ?	+	ND	ND	−	ND	−	+
B ♀ 4	−	+	−	S1+/env−	ND	ND	+	−	ND	ND	−	ND	−	+
B ♀ 5	−	+	−	S1+/env−	ND	ND	+	+	ND	ND	−	ND	−	+
B ♀ 6	+	ND	+	S1+/env+	*S1S1*	1,3,5	+	+	ND	ND	−	ND	+	+
B ♀ 7	−	+	−	S1+/env−	*S1S1*	1,5	+	−	ND	ND	−	ND	−	+
B ♀ 8	+	ND	+	S1+/env+	*S1S1*	1,3,5	+	+	ND	ND	−	ND	+	+
B ♂ 1	−	+	−	S1+/env−	*S1S1*	1,5	+	+	ND	ND	−	ND	−	+
B ♂ 2	+	ND	+	S1+/env+	*S1S1*	1,3,5	+	+	ND	ND	−	ND	+	+
B ♂ 3	+	ND	−	S1+/env+	*S1S1*	1,3,5	+	+	ND	ND	−	ND	+	+
B ♂ 4	−	+	−	S1+/env−	*S1S1*	1,5	+	−	ND	ND	−	ND	−	+
B ♂ 5	−	+	−	S1+/env−	*S1S1*	1,5	+	−	ND	ND	−	ND	−	+
B ♂ 6	+	ND	+	S1+/env+	ND	ND	+	+	ND	ND	−	ND	+	+
B ♂ 7	+	ND	+	S1+/env+	*S1S1*	1,3,5	+	+	ND	ND	−	MD	+	+
C ♀ 1	−	−	−	S1−/env−	*S3S3*	1,15,C	+	−	−	+	−	ND	−	+
C ♀ 2	−	−	−	S1−/env−	ND	ND	+	−	−	+	−	ND	−	+
C ♀ 3	−	−	−	S1−/env−	ND	ND	+	−	−	+	−	ND	−	+
C ♀ 4	−	−	−	S1−/env−	*S3S3*	1,15,C	+	−	−	+	−	ND	−	+
C ♀ 5	−	−	−	S1−/env−	ND	ND	+	−	−	+	−	ND	−	+
C ♀ 6	−	−	−	S1−/env−	ND	ND	+	−	−	+	−	ND	−	+
C ♀ 7	−	−	−	S1−/env−	*S3R*	1,5,D	+	−	−	+	−	ND	−	+
C ♀ 8	−	−	−	S1−/env−	ND	ND	+	−	−	+	−	ND	−	+
C ♂ 1	−	−	−	S1−/env−	*RR*	1,C,A	+	−	−	+	−	ND	−	+
C ♂ 2	−	−	−	S1−/env−	*S3S3*	1,15,C	+	−	−	+	−	ND	−	+
C ♂ 3	−	−	−	S1−/env−	ND	ND	+	−	−	+	−	ND	−	+
C ♂ 4	−	−	−	S1−/env−	*S3S3*	1,5,15	+	−	−	+	−	ND	−	+
C ♂ 5	−	−	−	S1−/env−	*S3S3*	1,15,C	+	−	−	+	−	ND	−	+
C ♂ 6	−	−	−	S1−/env−	ND	ND	+	−	−	+	−	ND	−	+
C ♂ 7	−	−	−	S1−/env−	*RR*	1,A,C	+	−	−	+	−	ND	−	+
D ♀ 1	−	−	−	S1−/env−	*RR*	1,A,C	+	−	−	+	ND	ND	ND	ND
D ♀ 2	−	−	−	S1−/env−	*RR*	ND	+	−	−	+	ND	ND	ND	ND
D ♀ 3	−	−	−	S1−/env−	*RR*	1,A,C	+	−	−	+	ND	ND	ND	ND
D ♀ 4	−	−	−	S1−/env−	ND	ND	+	−	−	+	ND	ND	ND	ND

Table 2. Continued.

Sample no.	R2 *TVB* analysis[A] Direct R2 assay	Indirect R2 assay	Serum R2 assay	Conclusion from the direct, indirect and serum R2 assays[B]	*TVB* SNP	Molecular analysis of *ALVE* loci Southern blot[C]	*ALVE* PCR primer sets[D] *ev1+*	*ev1−*	*ev21+*	*ev21−*	*ev2+*	*ev6+*	*ev3+*	*ev3−*
D ♀ 5	−	−	−	S1−/env−	ND	ND	+	−	−	+	ND	ND	ND	ND
D ♀ 6	−	−	−	S1−/env−	*RR*	1,A,C	+	−	−	+	ND	ND	ND	ND
D ♀ 7	−	−	−	S1−/env−	ND	ND	+	−	−	+	ND	ND	ND	ND
D ♀ 8	−	−	−	S1−/env−	ND	ND	+	−	−	+	ND	ND	ND	ND
D ♂ 1	+	ND	−	?	*RR*	1,B,C	+	−	−	+	ND	ND	ND	ND
D ♂ 2	+	ND	−	?	ND	ND	+	−	−	+	ND	ND	ND	ND
D ♂ 3	+	ND	−	?	*RR*	1,A,C	+	−	−	+	ND	ND	ND	ND
D ♂ 4	+	ND	−	?	*RR*	1,5,A	+	−	−	+	−	ND	−	+
D ♂ 5	(+)	ND	−	?	ND	ND	+	−	−	+	ND	ND	ND	ND
D ♂ 6	(+)	ND	−	?	*RR*	1,5,A	+	−	−	+	ND	ND	ND	ND
D ♂ 7	+/−	−	−	?	*RR*	1,A,C	+	−	−	+	ND	ND	ND	ND
D ♂ 8	(+)	ND	−	?	ND	ND	+	−	−	+	ND	ND	ND	ND
E 1	(+)	ND	ND	S1+/env+	*S1S1*	1,3,15	ND	ND	−	ND	−	ND	+	−
E 2	(+)	ND	ND	S1+/env+	*S1S1*	1,6,10,15	ND	ND	−	ND	−	ND	−	+
E 3	−	ND	ND	ID	*RR*	1,3,10,15	ND	ND	−	ND	−	ND	+	+
E 4	−	+	ND	S1+/env+	*S1S1*	1,15	ND	ND	−	ND	−	ND	−	+
E 5	(+)	ND	ND	S1+/env+	*S1S1*	1,15	ND	ND	−	ND	−	ND	−	+
E 6	−	+	ND	S1+/env−	*S1S1*	1	ND	ND	−	ND	−	ND	−	+
E 7	−	+	ND	S1+/env−	*S1S3*	1	ND	ND	−	ND	−	ND	−	+
E 8	+	ND	ND	S1+/env+	*S1S1*	1,16	ND	ND	−	ND	−	ND	−	+
E 9	(+)	ND	ND	S1+/env+	*S1S1*	1,3,6	ND	ND	−	ND	−	ND	+	+
E 10	(+)	ND	ND	S1+/env+	*S1S1*	1,3	ND	ND	−	ND	−	ND	+	+
F 1	−	+	ND	S1−/env+	*S1S1*	1,15	ND	ND	−	ND	−	ND	−	+
F 2	−	+	ND	S1−/env+	*S1S3*	1,15	ND	ND	−	ND	−	ND	−	+
F 3	−	−	ND	S1− and/or env−	*S3S3*	1,10	ND	ND	−	ND	−	ND	−	+
F 4	+	ND	ND	S1+/env+	*S1S3*	1,4,10,15	ND	ND	−	ND	−	ND	−	+
F 5	−	+	ND	S1+/env−	*S1S3*	1,15	ND	ND	−	ND	−	ND	−	+
F 6	ND clot	ND	ND	ID	*S1S1*	1,4,15	ND	ND	−	ND	−	ND	−	+
F 7	(+)	ND	ND	S1+/env+	*S1S3*	1,4,10,15	ND	ND	−	ND	−	ND	−	+
F 8	+	ND	ND	S1+/env+	*S1S1*	1,6,10	ND	ND	−	ND	−	ND	−	+
F 9	(+)	ND	ND	S1+/env+	*S1S1*	1,10	ND	ND	−	ND	−	ND	−	+
F 10	(+)	ND	ND	S1+/env+	ND	1,4,10,15	ND	ND	−	ND	−	ND	−	+

[A]In the direct R2 assay, + is a strong positive; (+) is weak positive; +/− is very weak positive, and − is a negative. ND = not done.

[B]The S1+ in the R2 conclusion column indicates the presence of the *TVB*S1* allele, and they are thus potentially susceptible to ALVE. The S1− indicates either the *TVB*S3*, *TVB*R*, or a *TVB*S3/R* heterozygote that is ALVE resistant. ID indicates insufficient data to make a determination. ? indicates inconsistent data (see Discussion).

[C]Southern blot indications A, B, and C represent unknown endogenous virus RFLP size patterns.

[D]When available, the PCR tests for ALVE were done to confirm the Southern blot analysis and to determine the heterozygous or homozygous nature of the inserts (4).

other segregating *ALVE* alleles would ensure this line remained p27 ELISA negative. Other lines are segregating for *ALVE*10, which is similar in phenotype to *ALVE*21. These lines are segregating for other *ALVE* loci that produce env-coded glycoproteins and the *TVB*S1* receptor.

The SPF breeder lines are often crossed to gain the F1 vigor needed to generate the number of fertile eggs required by the vaccine industry and regulatory testing agencies. In addition, CEFs from many embryos are pooled to generate the cultures necessary for vaccine production. The mixing of lines segregating for full-length *ALVE* loci and the *TVB*S1* receptor creates cultures with the potential to produce *ALVE* virus particles. *ALVE* is essentially innocuous, because it does not produce pathology in the chicken due to the weak nature of its viral promoters (LTR). However, the increased production of *ALVE* viral RNA produced by these mixtures increases the chance for recombination with contaminating exogenous ALV viruses with the potential of producing a new virus strain (15,21,22,30). In addition, the diploid packaging of full-length virus messages from different ALVs greatly enhances the recombination potential, and thus the expression of full-length endogenous viral RNA should be avoided (18,38,39). Production of cell-associated vaccines such as the Marek's disease vaccine requires that live CEFs be used as the inocula. The inoculation of CEFs expressing the ALVE virus due to cell-associated vaccines could generate a mild ALVE infection in *TVB*S1* ALVE env-coded glycoprotein-negative recipients. Upon hatch, these birds may become positive for the p27 ELISA assay and be discarded. In addition, regulatory agencies that monitor ALV contamination in biologicals require CEFs from defined genetic stocks that have the proper receptors for the virus being screened. Using the techniques described in this report the SPF egg producers could screen their lines and generate fine-tuned genetic stocks to better meet the needs of the vaccine industry and regulatory agencies.

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